Solution treatments that *enhance* a normal growth process offer a simple system for study of biochemical factors of morphogenesis in an intact organism under comparatively normal conditions. The mechanism by which the adenine mononucleotides, or other materials that may be found to favor a specific growth process, affect metabolism after penetration can be followed further by more refined techniques.

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*A RAPID METHOD FOR VIABLE CELL TITRATION AND CLONE PRODUCTION WITH HeLa CELLS IN TISSUE CULTURE: THE USE OF X-IRRADIATED CELLS TO SUPPLY CONDITIONING FACTORS**

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Studies of many aspects of the genetics and metabolism of animal cells are seriously handicapped by lack of a simple, effective technique for large-scale colony production from single cells. Thus it is not easily possible to develop a new population from the single-cell survivors isolated after the action of high-energy irradiation, virus invasion, or any of a variety of toxic drugs or nutritional stresses. This difficulty also makes almost impossible the preparation of mutant cell strains, a technique which has so enormously advanced studies of cell and virus genetics and biochemical transformations in microbial systems.[1] In addition, it is not possible to determine what fraction of any animal cell population is able to initiate multiplication, and therefore it becomes difficult to evaluate the mechanism of action of agents which change growth rates.

Earle and his associates[2] have studied quantitatively the minimum number of L-strain fibroblasts required in a given volume of medium in order to insure growth. Their careful investigations have emphasized the necessity for "conditioning" by the living cells of the nutrient media currently employed in tissue culture before multiplication can be supported. They were able to produce clones from a variety

of cells by a fairly exacting technique involving sealing of individual cells in capillary tubes, where diffusible metabolic products essential for multiplication remain in association with the cells. However, at most, only a few per cent of the single cells isolated by this method could be carried to the point of self-sustaining colony development. In attempting to develop a rapid method for growing clones from every viable cell, as is routinely accomplished with bacteria, we were impressed by the demonstration of Earle and his colleagues of the need to conserve diffusible cellular products, and so directed our efforts toward furnishing isolated cells with maximal amounts of these substances.

METHODS AND MATERIALS

The HeLa strain of epithelial cell obtained by Gey *et al.*[3] from a human cervical carcinoma was used throughout. These cells, initially supplied by the George Washington Carver Foundation, are maintained in this laboratory in continuous subculture. Growth medium of the following composition was employed: synthetic solution of amino acids, vitamins, and other growth factors,[4] 40 per cent; horse serum, 10 per cent; human serum, 20 per cent; Hank's solution, 30 per cent. Penicillin (100 units/cc) and streptomycin (100 μgm/cc) were routinely added. Cultures were incubated at 36.5° C. in an atmosphere of water-saturated air containing 3–5 per cent CO_2.

Suspensions of HeLa cells were prepared by trypsinization, according to standard techniques,[5] of the stock cultures which are grown on glass. Cell counts were made with a hemacytometer, and suspensions were diluted in nutrient medium to provide the requisite cell densities. These standardized suspensions were used in two types of experiments. In one procedure a series of approximately globular droplets, with volumes varying from 0.02 to 0.001 cc. and with progressively varying numbers of cells, was deposited on glass microscope slides, which were then placed in a Petri dish, empty except for cotton pledgets saturated with the growth medium, for humidity maintenance. These dishes were incubated, and the droplets subjected to daily microscopic count of cell numbers, from which growth rates were determined. In the other procedure, 0.05 cc. of standard cell suspensions was pipetted onto a piece of microscope slide (1.0 × 2.5 cm.) and spread with the tip of the pipette over the entire surface, to insure separation of the single cells. Microscopic examination of such slides confirmed that the cells so deposited lodge at widely separated points and in numbers to be expected within the uncertainty of sampling error. The slides were incubated for 5 hours, during which the cells attached to the glass. They were then placed in Petri dishes containing nutrient medium and nonmultiplying feeder cells, as described below, and reincubated. Fresh nutrient was exchanged for the old medium after approximately 3–4 days.

EXPERIMENTAL RESULTS

1. *Establishment of the Minimum Number of Cells Necessary To Initiate Sustained Growth in Nutrient Medium Alone.*—The first step consisted in determination of the minimum colony size which would permit uniformly successful subculture and regrowth to any desired cell number. It was found that colonies with as little as 25–50 cells could regularly be trypsinized and transferred to drops on new microscope slides or test tubes, where they would eventually yield confluent growth. By successive transfer from a slide colony to a test tube and then to a 1,000-cc. bottle, cell populations as great as 10^7 could be developed. To provide a margin of safety, the figure of 100 cells was selected as a minimum colony size from which routine subculture leading to any desired cell number could reliably be expected.

Next, experiments were undertaken to ascertain conditions under which isolated single cells would grow up to colonies of 100 or more. Varying numbers of cells, in droplets of nutrient medium of controlled size, were deposited on glass slides and incubated as described, in the thought that sufficiently small droplets might limit dilution of any essential, diffusible molecules as effectively as a capillary. However, the smallest drop size that could conveniently be manipulated contained a volume of 0.001 cc., which was still very much greater than that of the capillaries used by Earle. Such droplets regularly promoted growth past the stage of 100 cells only if they contained an initial inoculum greater than 11 cells (Table 1).

TABLE 1

GROWTH OF HeLa CELLS IN DISCRETE DROPLETS OF 0.001–0.0025 CC.

No. of Trials	No. of Cells in Original Inoculum	Per Cent of Tests Which Resulted in Cell Proliferation Past the 100 Mark*
56	1–5	18
12	6–10	66
32	11–50	100

* The criterion taken for unlimited growth was the development of a colony of 100 or more, since experience demonstrated that this number of cells could reliably be grown up to 10^7 by successive subculture in progressively larger containers.

The use of conditioned medium, as recommended by Earle, did not produce appreciable improvement. The medium was harvested after having supported a growing cell culture for 18–24 hours. Regardless of whether such conditioned medium was used full strength or diluted 1:1 with fresh medium, it produced no more success than fresh medium alone in promoting growth of small numbers of cells. It was concluded that, if the bulk of the cell population is really capable of reproducing and if the sole reason for failure of a single cell to initiate sustained multiplication is lack of a sufficient concentration of cell-manufactured, diffusible metabolites, some of these may have a half-life at 37° C. considerably less than 24 hours. Experiments were therefore designed to take account of this possibility.

2. *Growth of Clones from Single Cells Using X-irradiated "Feeder" Cells To Supply Conditioning Factors.*—Since the identity of the presumed short-lived, diffusible, conditioning factors was unknown, an attempt was made to provide a continuing supply of these by a large number of metabolizing cells which are themselves incapable of multiplication (feeder cells). If such growth-inhibited cells were arranged in close juxtaposition to a single multiplying cell, they could supply the latter with the necessary growth factors which should permit multiplication into a colony. Since high-energy irradiation operates to stop cell multiplication long before general metabolism is appreciably affected, this seemed an ideal agent for preparation of feeder cells to supply the needs of the immature clone.

Details of one experimental arrangement which gives successful results are as follows: 3×10^5 HeLa cells in 7 ml. of growth medium were pipetted into a 50-mm. Petri dish and incubated overnight to form the feeder layer. The following morning the medium was removed, replaced with 1 ml. of balanced saline, and the dishes exposed to a beam of hard X-rays (210–40 kv.; 15–20 ma.; 0.5-mm. Cu and 1.0-mm. Al filters). Doses varying between 5,000 and 40,000 roentgens appeared equally effective. After exposure, the original medium, centrifuged to remove any contained cells, was replaced and a sterile, U-shaped piece of Plexiglas laid over the feeder-cell layer. Two or three segments of glass microscope slide, on which vary-

ing aliquots of a standardized HeLa cell suspension had previously been spread, were then placed on the Plexiglas holder. The arrangement employed is shown in Figure 1.[6]

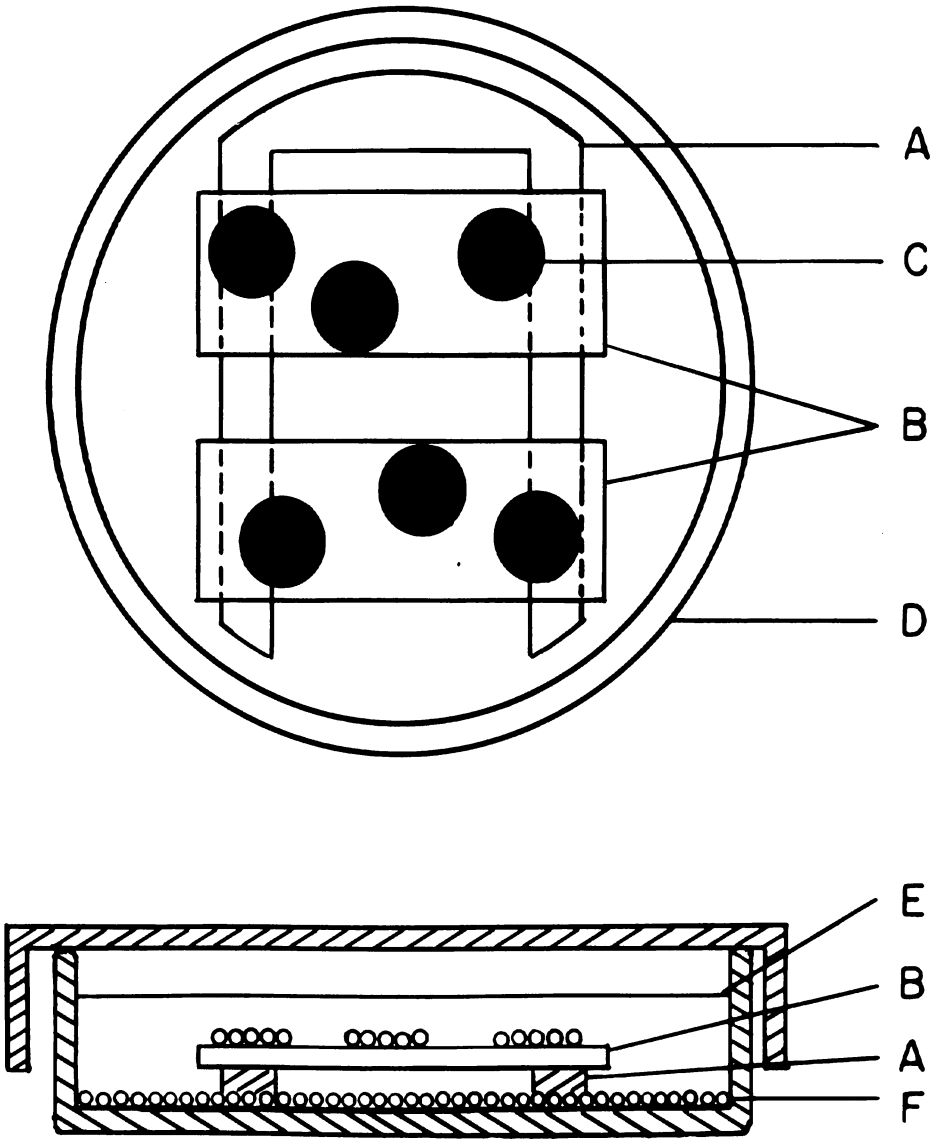
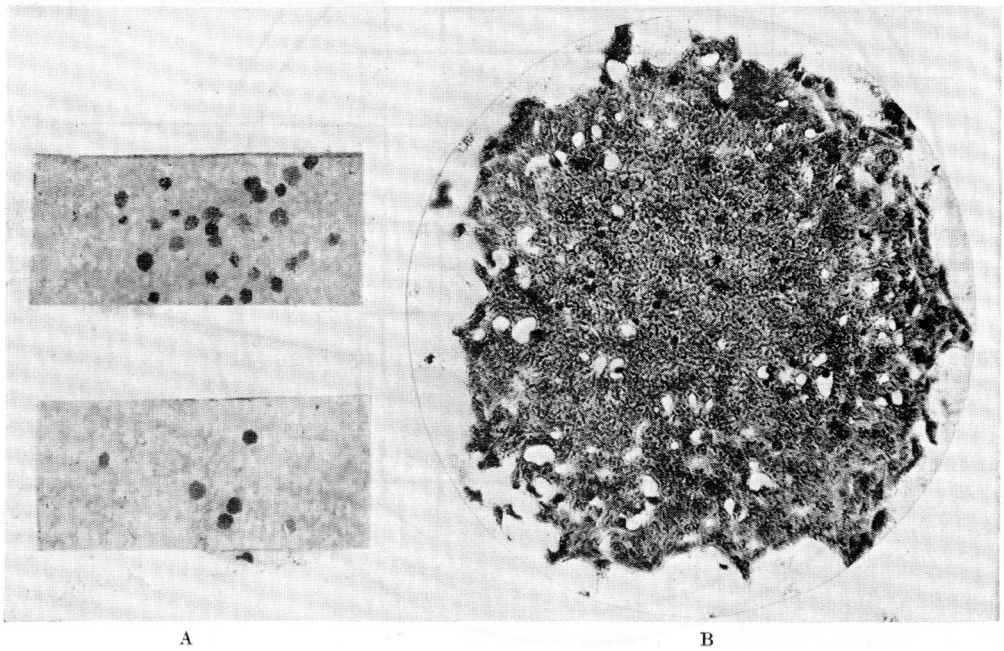


FIG. 1.—Schematic diagram of the arrangement used to grow clones of HeLa cells. Both the clones (which actually achieve a diameter of about 1 mm. in 8–10 days) and the cells are enormously exaggerated in size. *A*—U-shaped plastic holder supporting the microscope slides; *B*—segments of microscope slide on which the clones develop; *C*—clone; *D*—Petri dish; *E*—level of liquid nutrient medium; *F*—layer of feeder cells.

The plates were incubated for 8–10 days. Microscopic examination during this period revealed that the feeder layer undergoes little apparent change during the first 48–72 hours. Thereafter, this bottom cell layer degenerates, rounded forms appearing and debris accumulating in the supernate. After 4 days, only 10–20

per cent of the area of the Petri dish remains covered with recognizable cells, whereas, without irradiation, growth would be confluent.

During the static period of the feeder layer, the test cells on the microscope slide multiply steadily, reaching a clone population averaging about 800–2,000 and a diameter of about 1.0 mm. in 8–10 days. At this point the microscope slide can be removed and the colonies fixed, stained, and counted directly. A typical set of such colonies is illustrated in Figure 2, and Table 2 presents a comparison between



A B

FIG. 2.—*A*—Typical clones produced from single HeLa cells growing on glass slides above a feeder layer (×1.7). The top slide had 5 times as great an inoculum as the bottom slide. *B*—Photomicrograph of a typical clone (×50).

TABLE 2

COMPARISON BETWEEN THEORETICAL AND ACTUAL NUMBERS OF CLONES DEVELOPING ON MICROSCOPE SLIDES INOCULATED WITH SEPARATED HeLa CELLS AND GROWN AS DESCRIBED

Average No. of Single Cells Spread on Each Slide	No. of Clones Found	Mean and Standard Deviation
5	7, 3, 9, 5, 4, 2 9, 1, 2, 8, 7, 4	5.1 ± 2.8
10	10, 13, 10, 20 7, 9, 8, 10, 14 7, 5, 20 8, 9, 7, 11, 6 9, 11, 5, 6, 6, 10	9.6 ± 3.9
50	35, 44, 48 44, 43, 45, 44, 30	41.6 ± 5.6
100	77+, 68+	72.5+*

* This figure needs correction upward, because the colonies on these slides were so crowded as to make it certain that an appreciable number had been lost by coalescence.

the colony counts obtained and the average number of single cells contained in the aliquot deposited on the microscope slide. Correspondence between these numbers is as good as that obtained in bacterial assays. More than fifty such titrations

have been carried out, with no values falling outside the limits listed in the experiments of Table 2.

Experiments in which an attempt was made to secure similar results without irradiation of the feeder-cell layer were unsuccessful because cells from the bottom layer detach and are carried up to the test layer, where they may reattach and initiate colony formation. Thus, when an empty slide is placed on top of an unirradiated cell layer, as many as 150 colonies may develop which have originated from the bottom layer. Experiments which will be described later have demonstrated that the doses of X-irradiation employed inactivate the multiplication of 99.9 per cent of the feeder cells, so that, even if an occasional cell from the bottom finds its way to the test slide, it does not form a colony.

Periodic counts of the cells in the developing clones permit estimation of the doubling time, which was found to be 20 hours in young clones.

Preliminary results essentially similar were obtained with feeder cells produced by ultraviolet irradiation. However, unless quartz covers are available, the necessity for uncovering the Petri dishes during irradiation increases the risk of contamination.

DISCUSSION

The success of this titration permits the conclusion that almost all the HeLa cells obtained by trypsinization of a growing monolayer are capable of reproduction in an appropriate medium. The effectiveness of the feeder-cell principle also lends support to the idea that the sole reason for failure to obtain growth from single HeLa cells in the presence of large quantities of liquid is the loss of a short-lived, diffusible factor. New designs of simple growth arrangements for single cells, some of which eliminate the need for feeder cells, are being tested.

Since clones of these cells can easily be produced which exceed the critical size necessary for transfer to tubes and eventually to large bottles, it is possible to produce the large clonal populations needed for many types of genetic studies. Experiments with this technique on genetic and physiological actions of several agents are in progress. The applicability of this procedure to other types of animal cells is also under study.

SUMMARY

A simple method for production of clones from almost all the cells of a HeLa population is described. The technique can be used to titrate the number of cells capable of reproduction in a suspension and should find application in genetic and metabolic studies involving the action on animal cells of viruses, drugs, nutritional stresses, and mutagenic agents.

* Contribution No. 31. This study has been aided by a grant from the National Foundation for Infantile Paralysis and from the Damon Runyon Fund.

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[6] In our earliest experiments test cells and feeder cells were placed in the same layer. We wish to thank Dr. Leo Szilard, who suggested the more advantageous geometrical arrangement in which the test cells were placed on top of the layer of feeder cells.